Identification and cloning of class II and III chitinases from alkaline floral nectar of Rhododendron irroratum, Ericaceae

Identification and cloning of class II and III chitinases from alkaline floral nectar of *Rhododendron irroratum*, Ericaceae

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Abstract

Main conclusion

Class II and III chitinases belonging to different glycoside hydrolase families were major nectarins in *Rhododendron*

irroratum **floral nectar which showed significant chitinolytic activity.**

Previous studies have demonstrated antimicrobial activity in plant floral nectar, but the molecular basis for the mechanism is still poorly understood. Two chitinases, class II (Rhchi2) and III (Rhchi3), were characterized from alkaline *Rhododendron irroratum* nectar by both SDS-PAGE and mass spectrometry. Rhchi2 (27 kDa) and Rhchi3 (29 kDa) are glycoside hydrolases (family 19 and 18) with theoretical pI of 8.19 and 7.04. The expression patterns of Rhchi2 and Rhchi3 were analyzed by semi-quantitative RT-PCR. Rhchi2 is expressed in flowers (corolla nectar pouches) and leaves while Rhchi3 is expressed in flowers. Chitinase in concentrated protein and fresh nectar samples was visualised by SDS-PAGE and chitinolytic activity in fresh nectar was determined spectrophotometrically via chitin-azure. Full length gene sequences were cloned with Tail-PCR and RACE. The amino acid sequence deduced from the coding region for these proteins showed high identity with known chitinases and predicted to be located in extracellular space. Fresh *R. irroratum* floral nectar showed significant chitinolytic activity. Our results demonstrate that class III chitinase (GH 18 family) also exists in floral nectar. The functional relationship between class II and III chitinases and the role of these pathogenesis-related proteins in antimicrobial activity in nectar is suggested.

Keywords

Alkaline nectar
Chitinolytic activity
Class II chitinase
Class III chitinase
Glycoside hydrolase
Pathogenesis-related proteins

Abbreviation

GH Glycosyl hydrolase

Electronic supplementary material

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Introduction

Floral nectar is a rich source of sugar, amino acids, vitamins, organic acids, metal ions, and other metabolic components, which makes it potentially an excellent microbial growth medium (Nicolson and Thornburg 2007). Insects that visit flowers are non-sterile (Evans and Armstrong 2006), therefore as well as pollen, they can also transfer between flowers any microorganisms that they carry (Ferrari et al. 2006). Furthermore, flowers can remain open for several days, during which time their metabolically rich nectar would potentially allow microbial growth in close proximity to the plant's reproductive tract. However, despite this, infections of the gynoecium are relatively rare in plants. This implies there must be an active defense system in nectar to reduce such infections. A number of mechanisms have been reported with regards to antimicrobial properties of floral nectar, such as high levels of hydrogen peroxide (Carter and Thornburg 2004) and secondary compounds such as phenolics (Weston 2000). It is well known that floral nectar contains proteins (Carter and Thornburg 2000), which mainly comprise enzymes that protect the nectar from microbial infestation (Carter and Thornburg 2004). However, to date very few reports have characterized any nectar proteins directly involved in defense against microbes, with the exception of pathogenesis-related proteins, which protect extra-floral nectar from microbial infestation (Gonzalez-Teuber et al. 2009, 2010). One such group of proteins is chitinase (EC 3.2.1.14), which catalyze the hydrolysis of β-1,4 linkages in chitin, a polymer of N-acetyl-D-glucosamine.

Chitinases occur in many organisms such as plants, insects, fungi, bacteria, marine invertebrates and fish (Flach et al. 1992). From their amino acid sequences, chitinases are now classified into seven classes (classes I, II, III, IV, V, VI, and VII) (Neuhaus et al. 1996). According

to the family classification proposed by Henrissat and Davies (1997), chitinases belonging to classes I, II, IV, VI and VII are grouped into the glycosyl hydrolase family 19 (GH19), and those belonging to classes III and V are grouped into the GH18 family. Plants do not contain chitin, therefore plant chitinases may be involved in the defense of plants against chitin-containing pathogens (Collinge et al. 1993). Plant chitinases have been characterized with respect to their physiology and molecular structures and are implicated in defense mechanisms, especially against pathogen attacks on sensitive and unprotected organs (Collinge et al. 1993; Kasprzewska 2003; Grover 2012). Experimental evidence for chitinases acting as defense proteins has been obtained using transgenic plants that overexpressed chitinases and exhibited higher resistance to pathogens (Schlumbaum et al. 1986; Broglie et al. 1991; Minic 2008). Wagner et al. (2007) firstly detected chitinase (class IV, GH19 family) in pollination drop of several gymnosperm species, *Juniperus communis*, *Juniperus oxycedrus* and *Welwitschia mirabilis*. Gonzalez-Teuber et al. (2010) identified class I chitinase (GH19) in extrafloral nectar of *Acacia* species, and Escalante-Perez et al. (2012) identified classes III (GH18), IV (GH19), and V (GH18) in extrafloral nectar of *Populus trichocarpa*. Recently, a class I chitinase (GH19) and a class II chitinase (GH19) were detected in floral nectar of *Nicotiana attenuate* (Seo et al. 2013) and *Petunia* (Hillwig et al. 2011), respectively. On current evidence, GH19 chitinases are common in plant secretions on reproductive organs, pollination drops or floral nectar, whereas GH18 chitinases may be rarer. However, there has not been a detailed investigation into the roles of these proteins in preventing or limiting microbial growth in floral nectar.

Rhododendron is a genus which produces large insect-pollinated flowers with copious nectar. Moreover, the flowers can be open for up to 8 days (Primack 1985), and mostly employ a generalist pollination strategy, therefore they present ample opportunities for microbes to arrive and multiply. In the current study, therefore we focused on one large-flowered *Rhododendron* species, *R. irroratum* Franch, which is self-compatible and native to Yunnan in SW China (Zha et al. 2010). Here we characterize the nectar of this species with respect to general

characters such as sugar content and pH, and two specific factors that might be involved in direct defense from microbial attack: chitinases, and hydrogen peroxide.

Materials and methods

Rhododendron irroratum nectar collection, pH, hydrogen peroxide and sugar analysis

R. irroratum Franch. plants from the living collection in Kunming Botany Garden were used in this study. This species flowers from March to May, and flowers were examined during that period in 2009. Fresh nectar was collected from newly opened flowers with a pipette and autoclave tips. From each flower, ca. 20–50 µl of nectar was obtained. Fresh nectar from each individual was pooled and used as independent samples for sugar, total protein and chitinolytic activity analysis. Nectar samples were kept on ice and stored at −20 °C prior to use. Total sugars were estimated by the phenol–sulphuric acid method (Dubois et al. 1956) and with a digital refractometer (Atago PAL-1, Tokyo, Japan). The pH of fresh nectar from 55 flowers from 8 individual plants were tested by wide and narrow range pH test strips (Sigma). The level of hydrogen peroxide in pooled nectar from each individual plant was analyzed by a commercially available colorimetric assay kit (Beyotime, Jiangshu, China) and measurement was conducted as described in the manufacturer's manual.

Nectar protein quantification, concentration and electrophoresis

The protein content of collected nectar was determined according to Bradford (1976), using bovine serum albumin as standard. Fresh nectar protein was concentrated 20 times by ultra centrifugal filtering with Microcon YM-3 centrifugal filter units (cut-off 3000 Da; Millipore, Bedford, MA, USA). Distilled water was added after concentration to remove low molecular weight compounds by centrifugation.

Tricine sodium dodecyl sulfate-polyacrylamine gel electrophoresis (Tricine SDS-PAGE) was carried out on 12 % (w/v) self-poured

polyacrylamide gel following the method of Schagger and von Jagow (1987). Concentrated nectar protein samples were boiled in sample buffer (with and without 0.1 M dithiothreitol) for 5 min prior to gel loading. Two types of molecular weight protein markers (wide range and low molecular weight marker) were used as standards. Proteins were visualized by staining with Coomassie Brilliant Blue (CBB) G-250.

Mass spectrometry and protein identifications

For the identification of the target protein, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was used. Proteins were visualized with CBB-G250 after SDS-PAGE, and then the target bands were excised from the gel and washed with 25 mM NH_4HCO_3, 40 % (v/v) ethanol five times. The gel was cut into pieces and dehydrated with 1 ml of acetonitrile and dried in vacuo. Following this, 0.1 μg/ml trypsin in 25 mM NH_4HCO_3 was added to the gel pieces and incubated at 37 °C for 16 h. Peptide fragments were extracted from the gel pieces with 50 % (v/v) acetonitrile, 5 % (v/v) trifluoroacetic acid for 30 min. The extracts were dried in vacuo, dissolved in 5 μl of 50 % (v/v) acetonitrile, 0.1 % (v/v) trifluoroacetic acid, and subjected to an ABI 4700 proteomics analyzer (Applied Biosystems). Mass fingerprints of tryptic peptides dissolved in 5 μl of 50 % (v/v) acetonitrile, 0.1 % (v/v) trifluoroacetic acid were generated by MALDI-TOF–MS using an Applied Biosystems 4700 Proteomics Analyser with TOF/TOF optics in the MS mode. A Nd:YAG laser (355 nm) was used to irradiate the sample. The spectra were acquired in reflection mode in the mass range 700–3200 Da. Amino-acid sequences of the fragments were determined in MS/MS mode with DeNovo Explorer software (Applied Biosystems).

Nucleic acid extraction

Genomic DNA was extracted from young fresh leaves of *R. irroratum* individuals using a modified CTAB method (Kobayashi et al. 1998). Total RNA was isolated from fresh *R. irroratum* leaves, and separately from newly opened flowers' corolla basal nectar pouches (which

contain nectar glands), following the method of Jaakola et al. (2001). Extracted DNA and RNA quality and concentration were assessed using a nanodrop Spectrophotometer (ND-2000, Thermo Fisher Scientific).

Rapid amplification of cDNA 3′-end

Peptide sequences identified by mass spectrometry appeared to be chitinases of classes II and III. To amplify their DNA sequences, existing sequences for chitinase genes available from Genbank were examined and used to design gene specific primers: *Rhchi*2-3 for the class II chitinase gene, and *Rhchi*3-1F for class III chitinase gene (Table 1).

Table 1

DNA primers used for amplification of *Rhchi1* and *Rhchi3* from cDNA and gDNA

Primers	**Sequence[a] (5′–3′)**
Rhchi2-3	CCGTGGYCCCATCCAAATTWC
Rhchi3-1F	TGGGTKCARTTYTAYAAYARYCC
LAD1	ACGATGGACTCCAGAGCGGCCGCVNVNNNGGAA
LAD2	ACGATGGACTCCAGAGCGGCCGCBNBNNNGGTT
LAD3	ACGATGGACTCCAGAGCGGCCGCVVNVNNNCCAA
LAD4	ACGATGGACTCCAGAGCGGCCGCBDNBNNNCGGT
AC1	ACGATGGACTCCAGAG
Rhchi2-7	TTGCAGTCAAGRTTATTYCC
Rhchi2-F0	TACTAGCCAACCCAGACTTGGTCGCA
Rhchi2-F1	ACGATGGACTCCAGTCCGGCCACAATCCCCGAAACCC
Rhchi2-F2	GGATTGAGTGTGGAAAAGGGTCTACTAC
Rhchi2-R0	TGTCGAGACGAACGGTGAGATATTGAG
Rhchi2-R1	ACGATGGACTCCAGTCCGGCCTGCTGCAAGTGGGAAG
Rhchi2-R2	GAGAGAGAGAGATGGTGCTCACTGTG
Rhchi3-R0	CCCCTTTACAGCATCACTGTAACCAC

[a]IUPAC code for mix bases *M* a/c, *R* a/g, *W* a/t, *Y* c/t, *S* c/g, *K* g/t, *H* a/c/t, *V* a/c

Primers	Sequence[a] (5′–3′)
Rhchi3-R1	ACGATGGACTCCAGTCCGGCCACTTCCTTTGGAATGTA
Rhchi3-R2	TCGACTGTAACCATCGCGTCCATGAGT
Rhchi3-F0	TTCAAGGACTCATGGACGCGATGGT
Rhchi3-F1	ACGATGGACTCCAGTCCGGCCTCATGGACGCGATGGTT
Rhchi3-F2	CTGGCAACGGTTACATTCCAAAGGAAG
Rhchi2BR	GCCTTTGGTTATTGCAGTCAAG
Rhchi2BRTF1	ATGAGGATTTTGGCACTAATTTC
Rhchi3RTR1	GAGAGACCATTCAAGCCTCT
Rhchi3RTF2	ATGAAACTTCTTTCACCC
RhUBQF	AGAGGTGGTGTTGAACGATCG
RhUBQR	TCTCGCACTTATTACCGCACA
RhACTF	TCTTGATCTTGCTGGTCGTG
RhACTR	GGGCATCTGAATCTCTCAGC

[a]IUPAC code for mix bases *M* a/c, *R* a/g, *W* a/t, *Y* c/t, *S* c/g, *K* g/t, *H* a/c/t, *V* a/c

cDNA synthesis from *R. irroratum* corolla total RNA, and rapid amplification of cDNA 3′ end (RACE)-PCR were done with 3′-Full RACE Core Set kit (Takara, Dalian, China). RACE products were separated by electrophoresis on 1.5 % agarose gels. Specific bands were cut from the gel, purified and cloned into pMD 18-T vector (Takara) and sequenced in both directions with universal M13F (−47) and M13R (−48) primers on the vector. Verified 3′ end class II and III chitinase genes were named as *Rhchi2* and *Rhchi3*.

PCR on gDNA and cDNA

The 3′end of *Rhchi2* and *Rhchi3* gene cDNA sequence information was used for genome walking in the 5′ and 3′ directions using high-efficiency thermal asymmetric interlaced PCR (hiTAIL-PCR) methodology (Liu and Chen 2007). For the *Rhchi2* gene, primer

Rhchi2-7 was designed according to the end of the partial cDNA sequence. Primers Rhchi2-3 and Rhchi2-7 were used to amplify the partial region of *Rhchi2* from genomic DNA of *R. irroratum* using the following program cycle: 4 min at 94 °C (1 cycle); 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C (33 cycles); and 10 min at 72 °C (1 cycle). PCR products were purified, cloned and sequenced as above. Gene specific primers (Rhchi2-F0, F1, F2, R0, R1 and R2) were designed according to the sequence result. For *Rhchi3* gene, gene specific primers (Rhchi3-F0, F1, F2, R0, R1 and R2) were designed according to the partially obtained cDNA sequence by RACE. All primers used in this study including the arbitrary degenerate (LAD1–4, AC1) primers used for genome walking are listed in Table 1. The cycling conditions of Liu and Chen (2007) were strictly adhered to. The hiTAIL-PCR products were purified, cloned and sequenced as above. Overlapping DNA sequences were combined to make full-length *Rhchi2* and *Rhchi3* genes. New primers (Rhchi2BR and Rhchi2BRTF1 for *Rhchi2* gene, Rhchi3RTF2 and Rhchi3RTR1 for *Rhchi3* gene) based on the terminals of the deduced full length genes were used to amplify the genes from cDNA and gDNA and sequenced to verify the assembled results. All sequences generated in this study have been deposited in GenBank (Accession Nos. GU944515–GU944518).

Semi-quantitative RT-PCR

cDNA was synthesized from total RNA extracted from both the leaves and corolla nectar pouches as described above. Based on the cloned full length *Rhchi2* and *Rhchi3* sequence, gene specific primers (Rhchi2BR and Rhchi2BRTF1 for *Rhchi2* gene, Rhchi3RTF2 and Rhchi3RTR1 for *Rhchi3* gene) were used in reverse transcription-PCR (RT-PCR). The optimal amounts of cDNA and the number of PCR cycles corresponding to the exponential phase of the reaction were determined. PCR was performed using a PTC-200 thermocycler (MJ Research, Watertown, MA, USA) with the program: 2 min at 94 °C (1 cycle); 40 s at 94 °C, 40 s at 52 °C and 1 min at 72 °C (35 cycles); and 10 min at 72 °C (1 cycle). Housekeeping genes for *Rhododendron* (RhUBQ–ubiquitin and RhAct–actin) were selected as reference genes with *Rhododendron* special ubiquitin (RhUBQF/R) and actin (RhACTF/R) primers

(Nakatsuka et al. 2008; Peng et al. 2008), respectively, to investigate the relative expression of *Rhchi2* and *Rhchi3* in leaf and nectar pouches of *R. irroratum*. As a control for genomic DNA contaminations, all reactions were performed in duplicate with the control sample lacking reverse transcriptase. All RT-PCR products were separated by electrophoresis in a 1.5 % agarose gel and visualized after staining with ethidium bromide.

Computer analysis

The theoretical isoelectric point (pI) and molecular weight of mature Rhchi2 and Rhchi3 proteins were obtained using the Compute pI/Mw Web server (http://web.expasy.org/compute_pi/; Gasteiger et al. 2005). N-terminal signal peptide and cleavage sites were predicted using SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/; Petersen et al. 2011). Predictions of Rhchi2 and Rhchi3 subcellular localizations were performed by TargetP webserver using "PLANT networks" (http://www.cbs.dtu.dk/services/TargetP/; Emanuelsson et al. 2007) and YLoc (http://abi.inf.uni-tuebingen.de/Services/YLoc/) based on "YLoc-HighRes Plants model" (Briesemeister et al. 2010). The amino acid sequences of the mature peptide of Rhchi2 and Rchi3 were used to predict the disulfide bonds through the DiAminoacid Neural Network Application (DiANNA 1.1) Web server at http://clavius.bc.edu /~clotelab/DiANNA/ (Ferre and Clote 2006). Protein identification searches were performed in databases using software-tools found in "http://www.Expasy.org/." The sequence alignment was performed using Clustal X software (Larkin et al. 2007).

Activity staining of chitinases after SDS-PAGE

The chitinolytic activity of fresh nectar was carried out according to the method of Trudel and Asselin (1989). Glycol chitin obtained by acetylation of glycol chitosan (Sigma) was incorporated into the PAGE gel. Parallel SDS–PAGE was performed for visualization of chitinase activity with Calcofluor White stain (Fluka) and protein bands with CBB staining (Laemmli 1970). Nectar samples were not boiled before loading. To serve as controls, protein-free nectar was created by ultra

centrifugal filtering of fresh nectar using a Microcon YM-3. Into each well on the gel was loaded either 15 µl fresh *R. irroratum* nectar, 20 µg concentrated *R. irroratum* nectar protein, or 15 µl protein-free nectar. One nectar protein sample was run under reducing conditions with 0.1 M DTT in the sample buffer and the others were under non-reducing conditions. After electrophoresis, gels were incubated for 13–20 h at 37 °C in 50 mM Tris–HCl pH 8.9, 1 % Triton–X 100 (v/v), to remove SDS and promote chitinase activity against glycol chitin. First the gel was stained with 0.01 % Calcofluor White stain in 50 mM Tris–HCl pH 8.9 and the chitinase activity was determined by UV transilluminator. Areas of the activity bands with digested chitin were revealed as dark bands on a fluorescent background under the UV transilluminator and photographed. A replicate gel with the same samples stained with CBB G-250 was used for comparison of the positions of the protein bands.

Fresh nectar chitinolytic activity assay

Fresh nectar chitinolytic activity was tested using chitin azure (chitin covalently linked with Remazol Brilliant Violet 5R dye, Sigma) as substrate. For each individual experiment, 20 µl of fresh nectar from a single *R. irroratum* individual was incubated at 28 °C with 1 mg of chitin azure. This was done for each of six *R. irroratum* individuals, and for each individual, four separate samples were incubated for 2, 4, 8, or 24 h, making 24 treatments in total. In addition, three positive control samples were run using chitinase (0.01, 0.02, and 0.1 units) from *Streptomyces griseus* (Sigma, C6137) in 50 mM potassium phosphate (pH 6.0) incubated with 1 mg of chitin azure at the same reaction volume and for the same time periods. Protein-free nectar, 15 % glucose solution and sodium phosphate (50 mM, pH = 6.0) were included as reference samples.

Following incubation, the reaction mixture was cooled on ice for 10 min and centrifuged at 15,000*g* for 5 min. The absorbance of the supernatant was measured spectrophotometrically at 550 nm with nanodrop. Distilled water was used for instrument blanking.

Results

pH, total protein, total sugar and H_2O_2 of *R. irroratum* nectar

R. irroratum nectar was alkaline with a pH of 8.3 ± 0.29 (mean ± SD, $n = 55$). None of the pH of the nectar samples collected from 55 random selected flowers at different developmental stages was below 7.5, whereas floral nectar is generally slightly acidic (Nicolson and Thornburg 2007). The results of total sugar determination by two different methods were highly consistent, 14.6 ± 3.6 (ºBrix, $n = 8$) with a refractometer and 148 ± 53 mg ml^{-1} ($n = 8$) by phenol–sulphuric acid analysis, respectively. It showed that *R. irroratum* nectar was less concentrated for sugars than reported for other nectar (Baker and Baker 1983). Flowers with less concentrated nectar tend to be pollinated by birds and bats (Baker and Baker 1983); however, bees were observed as the main pollinator for *R. irroratum* flowers (Hong-Guang Zha, personal observation). The concentration of H_2O_2 in *R. irroratum* nectar was 30.1 ± 25.84 μM ($n = 15$), far less than the concentration reported in tobacco nectar which could be up to 4 mM (Carter and Thornburg 2000).

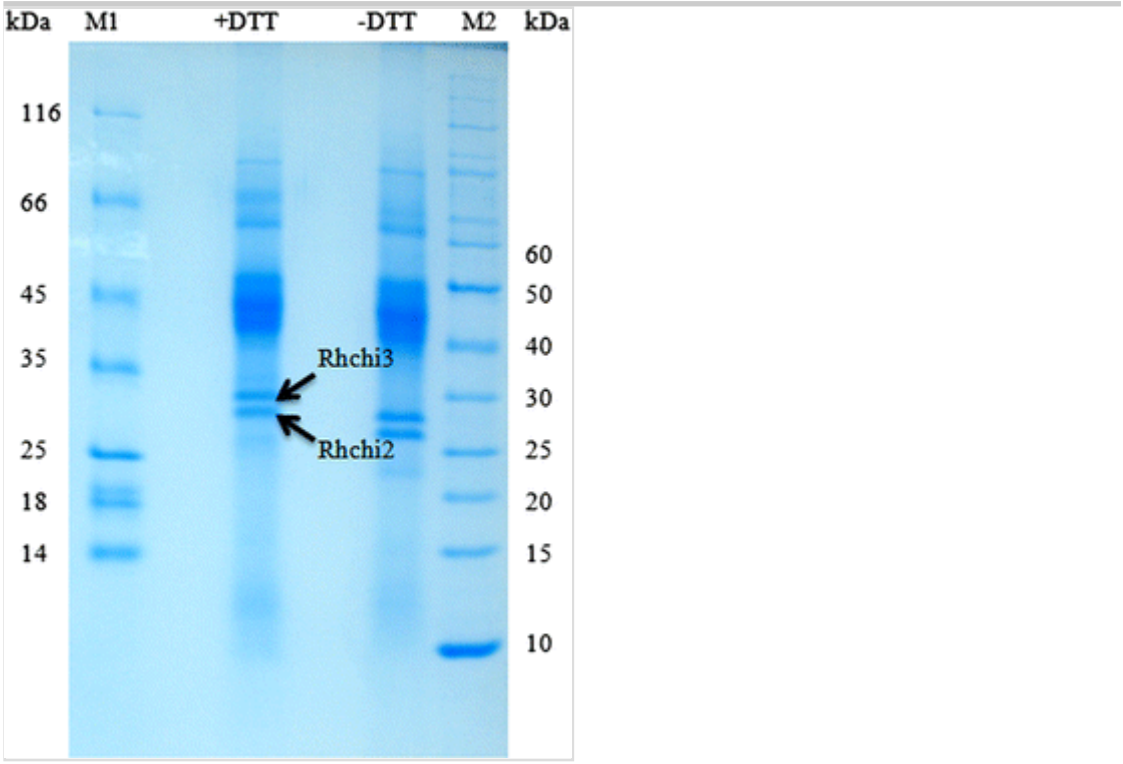
Nectar protein profile analysis by tricine SDS-PAGE

Concentrated nectar proteins were separated using tricine SDS-PAGE,yielding more than seven clearly distinguished bands as visualized by CBB G-250 stain under reducing (+DTT) or non-reducing (DTT) conditions (Fig. 1). Major protein molecular weight ranged from ca. 25 kDa to 100 kDa. Two clearly visible bands with apparent molecular weight 28 and 30 kDa, which were labeled as Rhchi2 and Rhchi3, respectively, were isolated and subjected to mass spectrometry analysis. Migration rates for both these proteins increased under non-reducing conditions, which indicated that disulfide bonds probably played an important role in their structure formation, and that both were likely to be monomers.

Fig. 1

Tricine SDS-PAGE of nectar proteins. *Lanes* 1 and 4 are different ranges of reference proteins with the molecular masses of the standards indicated; *lanes* 2 and 3 contain equal amount of concentrated nectar

protein under reducing (+DTT) and non-reducing conditions (−DTT). Protein bands were selected for mass spectrometry analysis and labeled as Rhchi2 and Rhchi3



Identification of Rhchi2 and Rhchi3 using mass spectrometry

Amino-acid sequences of internal regions of Rhchi2 and Rhchi3 were analyzed by MALDI-TOF–MS/MS and de novo sequencing analysis (Suppl. Fig. S1). MS/MS spectrum of two parent ions at *m*/*z* 1451.65 and 2642.2 for Rhchi2 were annotated by DeNovo Explorer, yielding the amino acid sequences “GFYTYEAFI(L)AAAK” and “TAL(I)WFWMTPQSPKPSSHDVI(L)TGR” for this peptide. Both the de-novo interpreted amino acids sequences matched well with the sequence of a typical class II chitinase from *Vaccinium corymbosum* (Kikuchi and Masuda 2009; Accession No. B9ZZZ5) and *Nepenthes khasiana* (Eilenberg et al. 2006; Accession No. Q6IVX4). Two parent ions at *m*/*z* 1110.5 and 1526.77 for Rhchi3 yielded the amino acid sequences “YGGI(L)ML(I)WDR” and “I(L)VNL(I)GFL(I)SAFGNFK” which matched the sequence of class III chitinase from *Sphenostylis stenocarpa* (Accession No. Q9XHC3), and *Nicotiana tabacum* (Lawton

et al. 1992; Accession No. P29061).

Cloning of the coding gene of Rhchi2 and Rhchi3

As described in Materials and methods, 3′ RACE and hiTAIL-PCR techniques were combined for the cloning of two full length chitinase genes. By the 3′ RACE method, two fragments containing poly A tail at the 3′ end of each of Rhchi2 and Rhchi3 were amplified with degenerate primers designed according to the MS identified sequence or a conserved region in the alignment of reported plant chitinase genes (data not shown). Sequence results were verified by Blastn, which showed high identity with reported Class II and III plant chitinase genes.

Based on the results of 3′ RACE, gene specific primers were designed for genome walking in the 5′ direction of the two genes. The full-length coding region of the Rhchi2 and Rhchi3 genes were amplified from cDNA and genomic DNA. For locations of gene specific primers used for cloning, see Fig. 2. Compared to the cDNA sequence, the genomic sequence of the Rhchi2 gene contained two introns, of 500 and 586 bp, in the coding region (Figs. 2a, 3). *Rhchi2* comprised 795 bp, encoding 264 amino acids. The first 19 amino acids function as a signal peptide as predicted by SignalP 3.0. The mature Rhchi2 protein had a predicted molecular mass of 26,355.35 Da and a fairly alkaline isoelectric point of 8.19. The genomic sequence of the Rhchi3 gene contained one 1474 bp intron in the coding region (Figs. 2b, 4). *Rhich3* comprised 891 bp, encoding 296 amino acids. The first 24 amino acids function as a signal peptide as predicted. The mature Rhchi3 protein had a predicted molecular mass of 29,525.19 Da and a neutral isoelectric point of 7.04. The predicted molecular mass of both Rhchi2 and Rhchi3 were consistent with SDS-PAGE results (Fig. 1) and with other reported plant class II and III chitinases (Hamel et al. 1997). The predicted signal peptides are likely required for both Rhichi2 and Rhchi3 for entry into the endoplasmic reticulum and later secretion via the endomembrane system (Vitale and Chrispeels 1992).

Fig. 2

Schematic representation of the genomic organization of Rhchi2 (**a**) and *Rhchi3* (**b**). *Rhchi2* shows the presence of three exons while Rhchi3 shows the presence of two (indicated by a *rectangular box*). Binding sites and directions of the primers are indicated by *arrows* and labeled as in Table 1 . Initiation codons and termination codons are shown

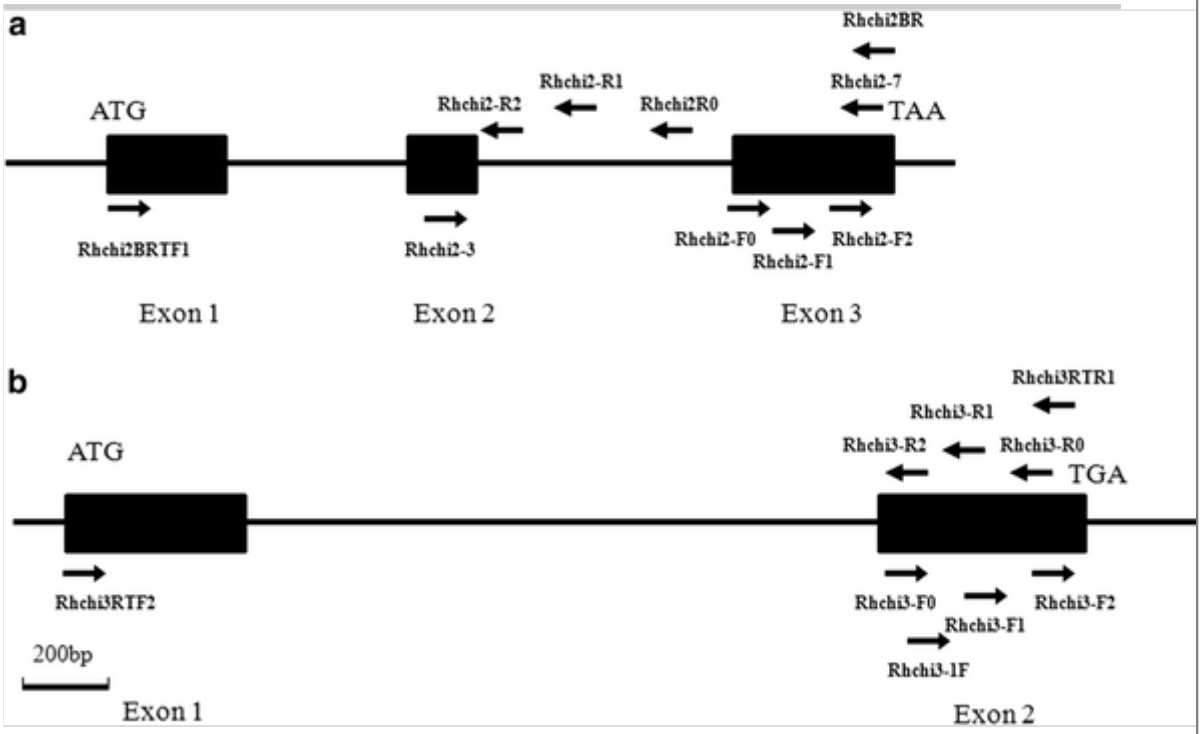


Fig. 3

Nucleotide (GenBank Accession No. GU944515 and GU944518) and deduced amino acid sequences of Rhchi2 (class II chitinase). The nucleotide sequence is numbered on the right. The deduced amino acids are shown in a *one-letter code* above the corresponding codons. The six conserved cysteine residues that presumably form three intramolecular disulfide bonds and the peptides identified by MS are indicated by *gray shading*. The stop codon, TATA box site and Poly (A) addition signal are indicated in *bold*. The stop codon is indicated with an *asterisk*. The open reading frame (ORF) of Rhchi2 encodes 264 amino acids (795 bp) with a predicted N-terminal signal peptide of 19 amino acids shown in *italics*

Fig. 4

Nucleotide (GenBank Accession No. GU944516 and GU944517) and deduced amino acid sequences of Rhchi3 (class III chitinase). The nucleotide sequence is numbered on the right. The deduced amino acids are shown in *one-letter code* above the corresponding codons. The catalytic glutamate residue in the chitinolytic active site is *boxed*. The six conserved cysteine residues that presumably form three intramolecular disulfide bonds and MS identified peptide are indicated by *gray shading*. The stop codon, TATA box site and Poly (A) addition signal are *indicated* in bold. The stop codon is indicated with an *asterisk*. The open reading frame (ORF) of Rhchi3 encodes 296 amino acids (891 bp) with a predicted N-terminal signal peptide of 24 amino acids shown in *italics*

AQ1

In addition, when Rhchi2 and Rhchi3 were analyzed by TargetP and YLoc programs, both proteins were predicted to enter the secretory pathway and to be located in the extracellular space. These predictions are in agreement with the hypothesis that Rhchi2 and Rhchi3 were secreted out from the nectary gland and presenting as soluble proteins in nectar.

Amino acid sequence analysis

A Blast search of the entire predicted Rhchi2 amino acid sequence revealed a significant affinity with chitinases and chitinase-like proteins of the GH 19 family, with the highest sequence homology of 85.2 % for *Vaccinium corymbosum* class II chitinase (Accession No. B9ZZZ5). Rhchi2 lacks the cysteine-rich domain (CRD) which is associated with chitin binding in class II chitinase classification. The carboxy-terminal extension (CTE) is also absent, which indicates that it is secreted to the apoplast while class I plant chitinases are targeted to the vacuole by means of a CTE signal (Hamel et al. 1997).

Rhchi3 showed a significant identity with class III plant basic endochitinase of the GH 18 family, with the highest identity (70 %) in the sequence of *Nicotiana tabacum* class III chitinase (Accession No. P29061). In addition, the two motifs conserved in class III chitinases, KVLLSLGGG and LDGIDFDIE including the catalytic glutamate (Esaka and Teramoto 1998), as well as an N-terminal signal peptide sequence like that found in other plant class III chitinases, were present. Based on the cloned sequences, we conclude that the Rhchi2 and Rhchi3 genes encode class II and III chitinase, respectively.

Based on the primary sequences of mature Rhchi2 and Rhchi3, DiANNA predicted three disulfide bonds connected for each protein as follows: Cys43–Cys105, Cys117–Cys126 and Cys225–Cys257 for Rhchi2; Cys44–Cys91, Cys74–Cys81 and Cys182–Cys211 for Rhchi3. Therefore, all the cysteines of Rhchi2 and Rhchi3 proteins are predicted to be involved in the disulfide bonds, which are conserved in class II

and III plant chitinase and considered to be crucial in maintaining tertiary structure formation (Beintema 1994; Huet et al. 2008). This result is consistent with the varied migration rate of Rhchi2 and Rhchi3 during SDS-PAGE under reducing and non-reducing conditions, because the reducing reagent DTT can disrupt disulfide bonds in proteins and results in a changed tertiary structure.

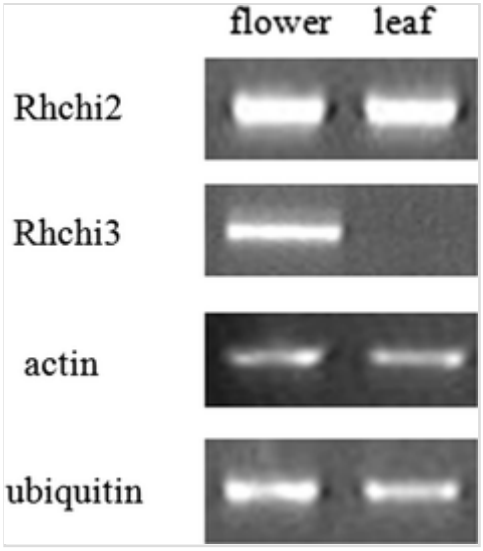
The identity between Rhchi2 and Rhchi3 based on amino acid sequence was only 12.4 %, which indicated no evolutionary relationship between them. The deduced amino acid sequences of Rhchi2 and Rhchi3 were compared separately with some known class II and class III plant chitinases from ExPASy server (Suppl. Fig. S2 and S3). Only sequences from species that have both class II and III chitinase sequences deposited in the database were considered, i.e., *Zea mays*, *Glycine max, Nicotiana tabacum, Vitis vinifera, Oryza sativa*, and *Gossypium hirsutum*. In cases where more than one chitinase homolog existed for the same species, the most similar one to Rhchi2 or Rchi3 was chosen for alignment. Both Rhchi2 and Rhchi3 show homology with other reported plant class II and III chitinases.

Rhchi2 and Rchi3 expression in leaves and flowers

The expression of the Rhchi2 and Rhchi3 in nectar pouches and leaves was examined using semi-quantitative RT-PCR with gene specific primers, and the constitutively expressed genes actin and ubiquitin were used as references (Fig. 5). Without reverse transcription, no amplified PCR products were generated (results not shown). Rhchi2 showed a similar pattern of expression in nectar pouches and leaves, while Rhichi3 only expressed in nectar pouches.

Fig. 5

RT-PCR analysis of *Rhchi2* and *Rhchi3* expression in flower (corolla nectar pouches) and leaf. Housekeeping genes, ubiquitin and actin, were used as control

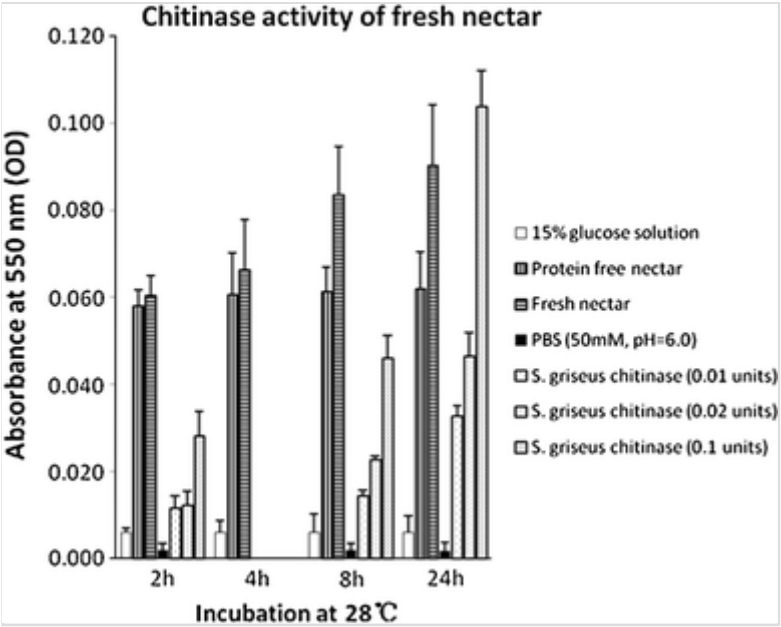


Determination of chitinolytic activity in *R. irroratum* nectar

Chitinase activity in fresh nectar was measured using chitin azure as the substrate and commercial chitinase from *Streptomyces griseus* as a positive control (Fig. 6). The chitinolytic activity in 20 µl of fresh nectar was almost 0.1 units S. *griseus* chitinase. Protein free nectar also showed some chitinolytic activity, but this was significantly lower than that of untreated fresh nectar, especially after an extended incubation time (Student's *t* test, $P < 0.001$). This indicated that unknown lower molecular mass compounds such as H_2O_2 in nectar might degrade chitin or disrupt the linkage between the dye and chitin.

Fig. 6

Chitinase activity of fresh nectar with chitin azure. Experiments were performed six times and the *small bars* represent standard errors. The chitinase activity of the fresh nectar was measured by the release of Remazol Brilliant Violet 5R from chitin azure and compared with the activity of *Streptomyces griseus* chitinase in 50 mM sodium phosphate buffer (PBS, pH = 6.0), which was used as a positive control

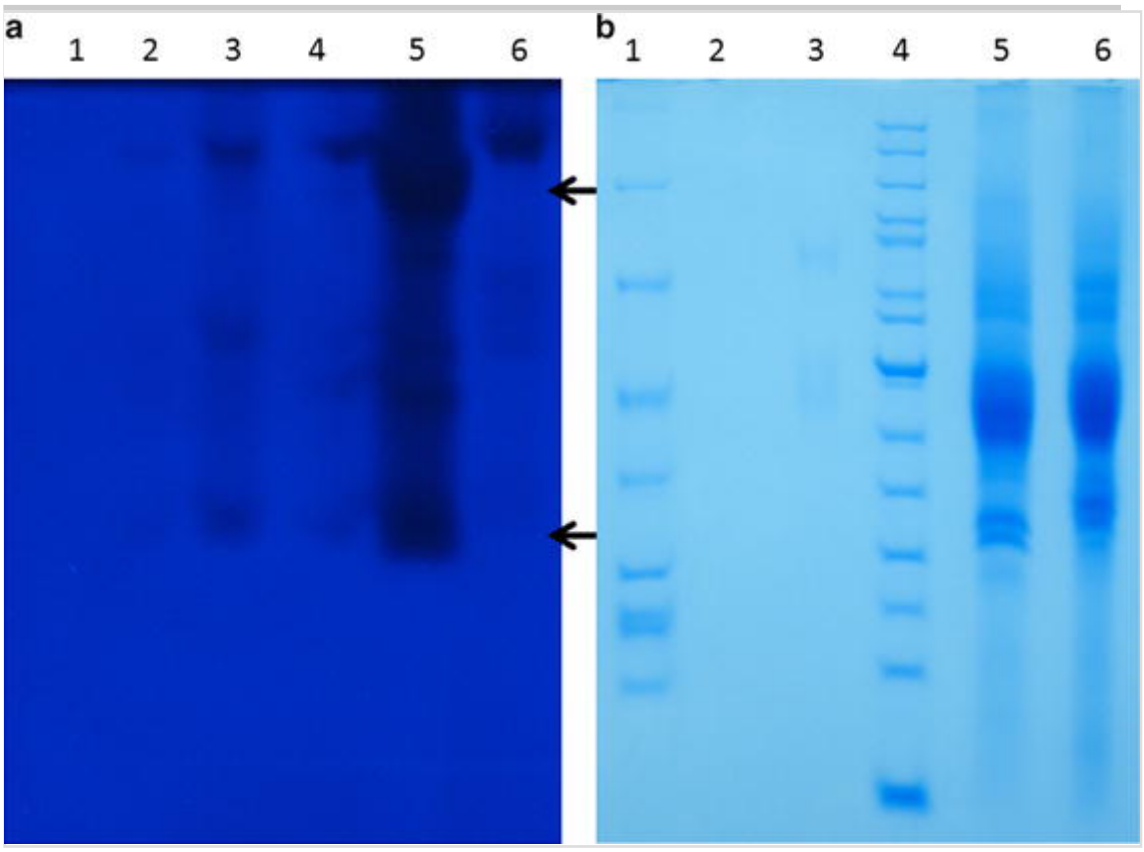


Both fresh nectar and concentrated nectar protein samples exhibited chitinolytic activity after SDS-PAGE (Fig. 7). No visible protein bands could be detected by CBB G-250 stain in the lane of the fresh nectar sample, which showed low protein concentration in that nectar sample. Nectar protein sample treated with DTT showed drastically reduced chitinolytic activity, which indicates a natural tertiary structure formation and that disulfide bonds are crucial for its chitinolytic activity. More than two positive bands with chitinolytic activity were visible in each of the fresh nectar and nectar protein lanes of the gel. The smallest band corresponds to the position of chitinases in the control gel. Another predominant band was at the position of ca. 100 kDa, which either indicates the presence of other chitinases in the nectar, or that major chitinases in the nectar were in trimer or tetramer form, having failed to dissociate under the SDS–PAGE conditions due to its SDS-resistant property and mild treatment before loading. Alternatively, glycol chitin in the zymogram gel could affect the chitinase migration rate during the electrophoresis or promote the chitinase monomers to form trimers or tetramers which have higher chitinolytic activity. Given that no evidence for other chitinases was found, and that both monomer and dimer formation were found together in *Bacillus thuringiensis* (Liu et al. 2010), we prefer the latter explanation, although the presence of other chitinases can not yet be

discounted.

Fig. 7

In gel chitinolytic activity assay. **a** SDS-PAGE with 0.02 % glycol chitin and stained with Calcofluor White stain, was photographed under UV. **b** Regular SDS-PAGE for nectar and concentrated nectar protein samples and stained with CBB G-250. For both gels, *lanes* 1, and 4, reference proteins M1 and M2, the same as described in Fig. 1; *lane* 2, protein free nectar; *lane* 3, fresh nectar; *lane* 5, concentrated nectar protein under non-reducing condition; *lane* 6, concentrated nectar protein under reducing condition. Bands containing chitinolytic activity are shown by *arrows*



Protein free nectar showed no chitinolytic activity in the gel, and no protein could be detected in it by CBB G-250 stain. This suggested that the chitinolytic activity in protein-free nectar is probably caused by compounds that cannot be removed by ultra centrifugal filtering, possibly due to small molecules. A likely candidate is H_2O_2, which has been demonstrated to be able to degrade chitosan (Chang et al. 2001).

A trace amount of chitinase might flow through, and be present in final filtrate of supposedly protein free nectar during the centrifugation process, but it could not contribute so much chitinolytic activity.

Discussion

GH18 and GH19 chitinases coexist in *R. irroratum* floral nectar

Chitinases are constitutively present in plants and developmentally regulated as well as tissue-specific (Hamid et al. 2013). Based on amino acid sequences, plant chitinases have been grouped into two families: GH family 18 and GH family 19. GH 19 chitinases contain globular domains, whereas GH 18 chitinases are characterised by 8 α-helices and 8 β-strands (Adrangi and Faramarzi 2013; Hamid et al. 2013). GH18 chitinases have a large distribution in organisms, including plants, bacteria, fungi, mammals, and viruses. However, GH 19 chitinase appears to be restricted to plants, fungi, and bacteria (Iseli et al. 1996; Udaya Prakash et al. 2010; Adrangi and Faramarzi 2013). Actinobacteria and purple bacteria might have acquired GH 19 chitinase genes from plants (Udaya Prakash et al. 2010). The chitinases of the two different families do not share amino acid sequence similarity, and have completely different 3-dimensional (3D) structures and molecular mechanisms (Hamid et al. 2013). Therefore, they are likely to have evolved from different ancestors. GH 18 and 19 chitinases exhibit different substrate specificities and use different hydrolytic mechanisms. The former carries out the hydrolysis of the β-1, 4-glycosidic linkage by means of a retaining mechanism, and the latter through an inverting mechanism (Hamid et al. 2013).

The present study firstly demonstrated that two different classes of chitinases (class II and class III) are both present in *Rhododendron irroratum* floral nectar, belonging to GH19 and GH18 family, respectively. Significant chitinolytic activity was detected in fresh *R. irroratum* nectar. The functional relationship between the class II and III chitinases in the nectar is still unclear. The physiological function of class II plant chitinases in plants is a matter of speculation; their

production could be induced by various environmental stresses and have dual functions (Kasprzewska 2003; Takenaka et al. 2009). Class II chitinases are generally extracellular, and can be detected in the apoplastic fluid or culture medium of protoplasts (Benhamou et al. 1990; Dore et al. 1991). They are not thought to be antifungal, either alone or in combination with other proteins, because of their lack of a cysteine-rich domain (Melchers et al. 1993). However, transgenic tobacco and wheat plants expressing a barley class II chitinase showed enhanced resistance to fungal infection (Jach et al. 1995; Oldach et al. 2001). The involvement of the cysteine-rich domain in antifungal efficacy has yet to be definitively characterized (Singh et al. 2007). Class II chitinase was reported from bark tissue of stems *V. corymbosum*, in the same family as *Rhododendron*, and was suggested to be involved in tolerance to low temperature in winter or unseasonably low temperature in spring (Kikuchi and Masuda 2009); it has a very high homology (77 %) with *Rhchi*2 in the primary structure. The altitude range of *R. irroratum* in Yunnan is 2000–3000 m (Chamberlain 1982), and it flowers from March to May, so it is possible that very low temperature in the morning during its flowering season could cause damage to the floral organ, and that antifreeze proteins in nectar could help protect against this.

Brunner et al. (1998) compared ten chitinases which functionally belong to all five classes in tobacco and drew the conclusion that the class III basic isoforms were the most efficient in inducing bacterial lysis while the class II chitinase was the least efficient. Furthermore, production of class III chitinases in rice can be induced by H_2O_2 (Park et al. 2004), which implies that their production might be an inducable response to pathogen attack. In alkaline *R. irroratum* nectar, class III chitinase (*Rhchi*3) and H_2O_2 (see below) may play a prominent role in antimicrobial activity, whereas the class II chitinase detected (*Rhchi*2) might be involved but probably has a main function as an antifreeze protein.

Antimicrobial mechanisms in *R. irroratum* nectar

Floral nectar from *R. irroratum* is alkaline, with a pH range of 7.5–8.8.

Most plant species have acidic nectar (Baker and Baker 1983; Nicolson and Thornburg 2007). To our knowledge the only reported exception is *Lathraea clandestina,* whose nectar is slightly acidic when freshly secreted (about pH 6.5), but turns highly alkaline (pH 11.5) in aged flowers (Prŷs-Jones and Willmer 1992). Therefore, our finding is the first report of nectar above pH 7.5 in freshly opened flowers, or consistently above 7.5. Given that low pH might be an important mechanism for antimicrobial activity in honey (Molan et al. 1997), the same might apply in nectar. It is reasonable that some extreme acidic nectar have similar mechanisms to resist microbial attack. If so, where species do not have acidic nectar, other antimicrobial mechanisms would be necessary.

So far, two protective strategies have been well demonstrated with regard to the antimicrobial property of nectar, H_2O_2 in tobacco floral nectar and pathogenesis-related (PR) proteins in the extraflora nectar (EFN) of *Acacia* species (Carter and Thornburg 2004; Gonzalez-Teuber et al. 2009, 2010; Heil 2011, 2015; Nocentini et al. 2015). In the present study, both H_2O_2 and chitinases were detected in *R. irroratum* nectar. The concentration of H_2O_2 varied between 18 and 104 μM with an average of 30 μM; this is less than was reported for tobacco nectar (Carter and Thornburg 2000), but matches the range of concentrations (10–100 μM) that are normally toxic to cells (Halliwell and Gutteridge 1999). Indeed, lower concentrations than this can induce random degradation of partially deacetylated chitin and chitosan (Chang et al. 2001). The presence of H_2O_2 therefore provides a possible explanation for the chitinolytic and antifungal activity we detected in *R. irroratum* nectar from which proteins have been removed. A high level of H_2O_2 in rich nectar is hypothesized to maintain it in an axenic state either by inhibiting the growth of microorganisms or by directly killing them (Carter and Thornburg 2004). However, a high level of H_2O_2 in nectar could also become deleterious due to its instability in the presence of metal ions and the consecutive generation of free hydroxyl radicals (Gonzalez-Teuber et al. 2010); if so there must be clear benefits from H_2O_2 in nectar to balance these costs, and the concentration of it may be a tradeoff between costs and benefits.

The wide variation in H_2O_2 concentrations between flowers observed in the current study, and to a greater extent (4 μM–20 mM) by Carter and Thornburg (2000), indicates that H_2O_2 production probably varies between flowers, according to conditions. The early phase of plant response to either pathogen- or plant-derived elicitors is usually accompanied by the production of H_2O_2 (Alvarez et al. 1998). H_2O_2 mediates defense responses that could be induced by chitosans of different molecular weights in rice (Lin et al. 2005). It is suggested that the production of H_2O_2 in nectar might be induced by infection with pathogens, and maintains a low level during the normal stage with no harm to the tender tissue in floral organ. Besides any direct effects upon pathogenic organisms, H_2O_2 also appears to be involved in signaling for other pathogen responses. H_2O_2 has been shown to induce pathogen response proteins including chitinase in plants (Chamnongpol et al. 1998; Park et al. 2004), and acts as a messenger to induce or increase the transcription of genes related to the plant immune system which could promote specific chitinase productivity (Akimoto et al. 2000).

In the present study, fresh untreated nectar showed higher chitinolytic and antifungal activity against *Botrytis cinerea* than did protein free nectar, especially after extended incubation times, implying both an effect from H_2O_2 and a role for proteins such as chitinases in chitinolysis (data not shown). Therefore, it is hypothesized that in alkaline *R. irroratum* nectar both protective strategies with regard to the antimicrobial property apply: H_2O_2 acts as the first line of defense, both reacting with invading microbes directly and inducing the production of PR proteins such as chitinases (most probably *Rhchi*3), which play an important role in restricting microbial growth later on. However, the possible involvement of other nectarin-generated compounds could not be excluded.

It is interesting that we could not detect chitinase by SDS-PAGE in floral nectar of *Rhododendron delavayi*, a closely related species of *R. irroratum* with acidic floral nectar. The concentration of H_2O_2 and chitinolytic activity in fresh *R. delavayi* nectar was one tenth lower than in *R. irroratum*'s nectar (data not shown). Obviously, pronounced

different antimicrobial mechanisms in nectar are used even by closely related species which indicates that rapid evolution occurs.

Several classes chitinase (mainly belonging to GH family 19) were detected as major nectarins in floral or extra-floral nectar of various plant species as described above. Besides Rhchi3 in this study, one GH family 18 chitinase (class III) was also detected in extra-floral nectar of *Populus trichocarpa* (Escalante-Perez et al. 2012). Even though floral and extrafloral nectar are quite different in function, position, origin, nectar consumers and other aspects (Pacini and Nicolson 2007; Heil 2011, 2015), both have adopted similar antimicrobial mechanism using chitinases in the protection. Chitinases of both families that differ in activity, chitin binding property, substrate specificity, and catalytic mechanism presenting in nectar might allow plant to match different requirements, such as various fungi or bacteria invading into nectar.

In future work we aim to test the individual roles of these proteins and investigate the possibility that other proteins are also involved in anti-microbial activity.

Author contribution statement HGZ and HS conceived and designed research. HGZ, XC and HXZ conducted experiments. HGZ and RM analyzed data. HGZ and RM wrote the manuscript. All authors read and approved the manuscript.

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Compliance with ethical standards

Conflict of interest The authors have declared that no competing interests exist.

Ethical statement Our work complies to the ethical rules applicable for this journal.

Electronic supplementary material

Below is the link to the electronic supplementary material.

Supplementary Fig. S1

MS spectra of identified proteins and fragments. **a** Rhchi2 MS spectra. **b** MS/MS spectra of *m/z* 1451.65, "GFYTYEAFI(L)AAAK" fragment in Rhchi2. **c** MS/MS spectra of *m/z* 2642.2, "TAL(I)WFWMTPQSPKPSSHDVITGR" fragment in Rhchi2. **d** Rhchi3 MS spectra. **e** MS/MS spectra of *m/z* 1110.5, "YGGI(L)ML(I)WDR" fragment in Rhchi3. **f** MS/MS spectra of *m/z* 1526.77, "I(L)VNL(I)GFL(I)SAFGNFK" fragment in Rhchi3 (TIFF 2255 kb)

Supplementary Fig. S2

Comparison of Rhchi2 amino acid sequence with that of six class II plant chitinase homologues. Amino acids, which are completely conserved are marked with asterisks, and the highly conserved amino acids are marked with dots or double dots. -, gap left to improve alignment. Numbers refer to amino acid residues at the end of the respective lines. Species names are abbreviated at the left and represent with accession number: Zmchi2 (*Zea mays*, B6SZC6), Gmchi2 (*Glycine max*, C6TNB0), Ntchi2 (*Nicotiana tabacum*, Q9ZWS3), Vvchi2 (*Vitis vinifera*, A5AT00), Qschi2 (*Oryza sativa*, Q7XCK6), Ghchi2 (*Gossypium hirsutum*, P931545) (DOC 105 kb)

Supplementary Fig. S3

Comparison of Rhchi3 amino acid sequence with that of six class III plant chitinase homologues. Amino acids, which are completely conserved are marked with asterisks, and the highly conserved amino acids are marked with dots or double dots. -, gap left to improve alignment. Numbers refer to amino acid residues at the end of the respective lines. Species names are abbreviated at the left and represent an accession number: Zmchi3 (*Zea mays*, B4G1T3), Gmchi3 (*Glycine max, C6T8G2)*, Ntchi3

(*Nicotiana tabacum, P29061)*, Vvchi3 (*Vitis vinifera, Q84S31)*, Qschi3 (*Oryza sativa*, *Q84ZH2*), Ghchi3 *(Gossypium hirsutum, A2TJX5)* (DOC 110 kb)

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